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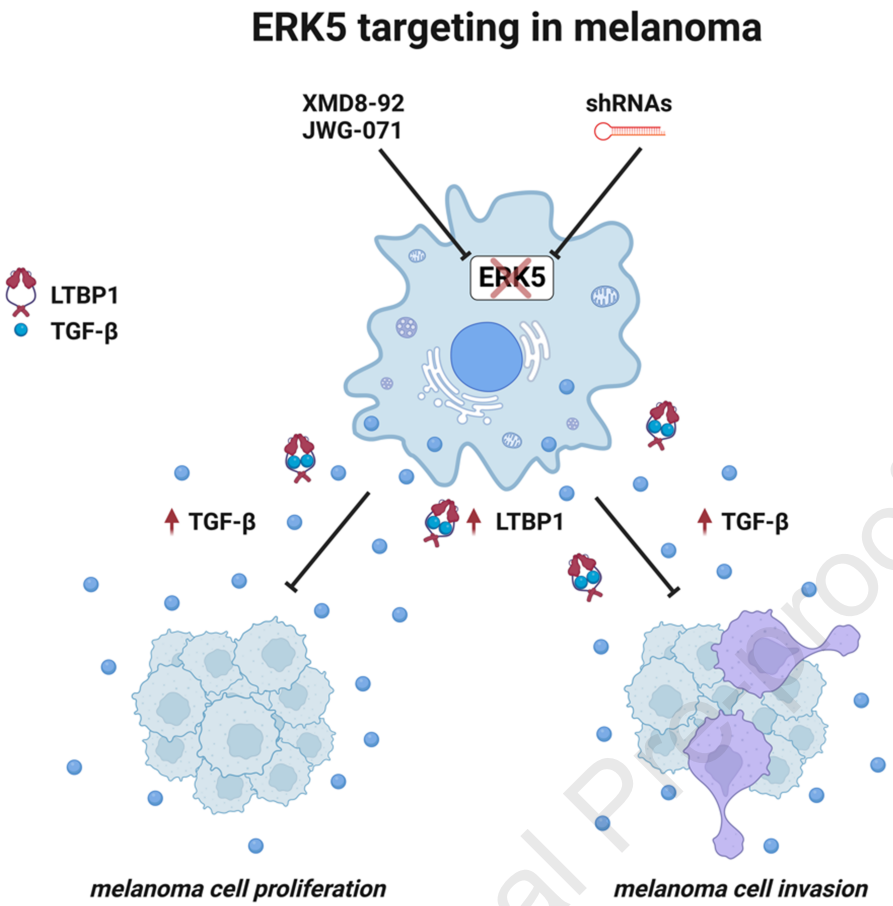

# Latent-transforming growth factor β-binding protein 1/Transforming growth factor β1 complex drives antitumoral effects upon ERK5 targeting in melanoma

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**Abstract**

Melanoma is the deadliest skin cancer, with a poor prognosis in advanced stages. Available treatments have improved the survival, although long-term benefits are still unsatisfactory. The mitogen-activated protein kinase ERK5 promotes melanoma growth, and ERK5 inhibition determines cellular senescence and the senescence-associated secretory phenotype. Here, latent-transforming growth factor β-binding protein 1 (LTBP1*)* mRNA was found to be upregulated in A375 and SK-Mel-5 BRAFV600E melanoma cells after ERK5 inhibition. In keeping with a key role of LTBP1 in regulating transforming growth factor β (TGF-β), TGF-β1 protein levels were increased in lysates and conditioned media of ERK5-knock down (KD) cells, and were reduced upon LTBP1 KD. Both LTBP1 and TGF-β1 proteins were increased in melanoma xenografts in mice treated with the ERK5 inhibitor XMD8-92. Moreover, treatment with conditioned media from ERK5-KD melanoma cells reduced cell proliferation and invasiveness, and TGF-β1-neutralizing antibodies impaired these effects. In silico datasets revealed that higher expression levels of both LTBP1 and TGFB1 mRNA are associated with better overall survival of melanoma patients, and that increased LTBP1 or TGF-β1 expression proved a beneficial role in patients treated with anti-PD1 immunotherapy, making unlikely a possible immunosuppressive role of LTBP1/TGF-β1 upon ERK5 inhibition. This study, therefore, identifies additional desirable effects of ERK5 targeting, providing evidence of an ERK5-dependent tumor suppressive role of TGF-β in melanoma.

## Introduction

Malignant melanoma is one of the most aggressive skin cancers, and its incidence is increasing worldwide. Early-stage disease can be cured in the majority of cases by surgical excision, while late-stage melanoma is still a highly lethal disease [1]. Common genetic alterations associated with melanoma include mutations in BRAF (50-60%), NRAS (20-25%) and NF1 (14%) that hyperactivate the mitogen-activated protein kinase (MAPK) ERK1/2, thus supporting sustained cell proliferation [2]. Development of BRAF- and MEK1/2-targeting drugs and immunotherapy have greatly increased the survival of melanoma patients [3]. However, intrinsic or acquired resistance to the former as well as the lack of responsiveness to the latter limit the benefits of available therapies [4,5].

ERK5 (also referred to as big mitogen-activated protein kinase 1, BMK1), the last discovered member of conventional MAPKs, is involved in cell survival, proliferation and differentiation of several cell types [6], and plays a relevant role in the biology of cancer, including melanoma [7,8,9]. ERK5 activation is achieved through MEK5-dependent or -independent phosphorylation that stimulates ERK5 nuclear translocation, a key event for cell proliferation [10,11]. On the other hand, a recent report showed that, upon ERK5 inhibition, melanoma cells undergo cellular senescence, and produce a number of soluble mediators (namely CXCL1, CXCL8 and CCL20) typically involved in the senescence-associated secretory phenotype (SASP) that slow down the proliferation of melanoma cells [9,12].

Accumulating evidence points to the involvement of transforming growth factor β (TGF-β) in cellular senescence [13]. TGF-β secretion and activation is regulated by its association to latent-transforming growth factor β-binding protein 1 (LTBP1) [14,15]. The roles normally played by TGF-β signaling are to control proliferation, differentiation and other functions in most cells. These roles are highly context-dependent, and TGF-β appears to induce even opposite effects in different contexts [16]. Regarding melanoma in particular, the role of tumor suppression versus tumor promotion of TGF-β has been scarcely addressed [17]. This paper identified a tumor suppressive

role for LTBP1/TGF-β among the antitumoral outcomes of ERK5 inhibition, that could be exploited for future therapeutic strategies in melanoma.

## Material & Methods

*Cells and cell culture*

A375$^{BRAFV600E}$ (RRID:CVCL_0132) [18] and SK-Mel-2$^{NRASQ61R}$ (RRID: CVCL_0069CVCL_0069) [19] melanoma cells were obtained from ATCC; SK-Mel-5$^{BRAFV600E}$ (RRID:CVCL_0527) melanoma cells [19] were kindly provided by Dr. Laura Poliseno (CRL-ISPRO, Pisa, Italy); SSM2c melanoma cells have been described elsewhere [20]. Cells were maintained in DMEM with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L glutamine, 50 U/mL penicillin and 50 mg/mL streptomycin (Euroclone, Milano, Italy). Cell lines are authenticated yearly (BMR Genomics, Padua, Italy) by STR profiling using Promega PowerPlex Fusion System Kit (Promega Corporation, Madison, WI, USA). Presence of Mycoplasma was periodically tested by PCR.

*Drugs*

ERK5 inhibitors XMD8-92 [21] and JWG-071 [22] were from MedChemExpress (Monmouth Junction, NJ, USA). Cell cycle inhibitor L-mimosine was from Sigma-Aldrich (St Louis, MO, USA).

*Cell lysis and Western Blot*

Total cell lysates were obtained using Laemmli Buffer or RIPA buffer as reported previously [23]. Immunoprecipitation (IP) was performed by incubating 2 mg of CM proteins with the anti-TGF-β antibody and 20 µL of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, USA) for 24 hours at 4 °C. Immunocomplexes were then washed for three times and proteins eluted using Laemmli Buffer. Proteins were separated by SDS-PAGE and transferred onto Amersham Protran nitrocellulose

membranes (GE Healthcare, Chicago, IL, USA) by electroblotting. Infrared imaging (Odissey, Li-Cor Bioscience, Lincoln, NE, USA) was performed. Images were quantified with ImageJ 1.53k software (https://imagej.net/ij/; Last access 18/01/2024). The list of the antibodies is in Table 1.

*RNA interference*

A375 and SK-Mel-5 cells were transduced with control non-targeting shRNA (shNT) or ERK5-specific shRNAs (shERK5-1 and shERK5-2) (Table 2) as previously reported [23]. Transduced cells were selected with 2 μg/mL puromycin for at least 72 hours. Fourteen days after lentiviral transduction, medium was replaced with DMEM/10% FBS, and CM were harvested after 72 hours. For siRNA inhibition studies, the cells were transfected with human LTBP1 siRNAs (SASI_Hs01_00187276 and SASI_Hs01_00168991) or negative control siNT (SIC001) from Sigma-Aldrich at a final concentration of 100 nM using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. 72 hours after transfection, cells were harvested for protein extraction and additional analysis.

*Measurement of cell viability, cell cycle phase distribution and cell death*

The number of viable cells in culture was evaluated by counting trypan blue-positive and negative cells with a hemocytometer. Cell cycle phase distribution (propidium iodide staining) was estimated by flow cytometry using a FACSCanto (Beckton & Dickinson, San Josè, CA, USA) as previously reported [24]. Dead cells were evaluated by flow cytometry using a FACSCanto (Beckton & Dickinson). AnnexinV-positive and Annexin-V-negative/PI-positive cells were measured using Annexin-V-FLUOS Staining Kit (Sigma-Aldrich), as previously reported [24].

*Transcriptomic analysis*

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany), and mRNA expression was evaluated with Affymetrix Clariom-S Human Genechip following the manufacturer's

instructions. Transcriptome analysis console (TAC) software was used (fold change>1.5/<1.5 and $p \leq 0.05$) to identify differentially expressed genes (DEG).

*Patients*

Analysis of the relationship between LTBP1 and TGFB1 mRNA expression and overall survival (OS) of melanoma patients was carried out using the publicly available SKCM data set from The Cancer Genome Atlas (TCGA PanCancer Atlas) on cBioPortal for Cancer Genomics (https://www.cbioportal.org; last access: October 10th, 2023 [25,26]). The same database was used to verify the correlation between LTBP1 and TGFB1 mRNA. Analysis of the relationship between LTBP1 and TGFB1 mRNA expression and outcome OS and DFS of melanoma patients treated with anti-PD1 therapy was carried out using the open access database Kaplan-Meier plotter (http://www.kmplot.com; last access: October 10th, 2023 [27]). Expression of LTBP1 in normal, primary and metastatic tumors was obtained from TCGA dataset on TNMplot database (http://www.tnmplot.com; last access: October 10th, 2023 [28]).

*Quantitative real-time PCR (qPCR)*

Total RNA was isolated using TriFast II (Euroclone). cDNA synthesis was carried out using ImProm-II Reverse Transcription System, while qPCR was performed using GoTaq qPCR Master Mix (Promega Corporation). QPCR was performed using CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Expression levels were determined by qPCR with the primers: Forward: 5′-TGAATGCCAGCACCGTCATCTC-3′ and reverse: 5′-CTGGCAAACACTCTTGTCCTCC-3′ for LTBP1. mRNA expression was normalized to: Forward: 5’-GTCTCCTCTGACTTCAACAGCG-3’ and reverse: 5’-ACCACCCTGTTGCTGTAGCCAA-3’ GAPDH mRNA and: Forward: 5’-ACCCGTTGAACCCCATTCGTGA-3’ and reverse: 5’-GCCTCACTAAACCATCCAATCGG-3’ for 18S mRNA.

*Cell viability assay*

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well plate in DMEM/10% FBS. After 24 hours, medium was replaced with CM and cells were further incubated for 72 hours. MTT (0.5 mg/mL) was added during the last 4 hours. Plates were read at 595 nm using a Microplate reader-550 (Bio-Rad). For neutralization experiments, control isotype IgG or neutralizing antibodies (Table 1) were added to CM prior to administration to cells.

*Immunohistochemistry*

Formalin-fixed paraffin-embedded sections from archival xenografts established with A375 cells from XMD8-92 (25 mg/kg)- or vehicle (2-hidroxypropyl-β-cyclodextrin 30%)-treated mice were used [8]. Experiments had been approved by the Italian Ministry of Health (authorization no. 213/2015-PR) and were in accordance with the Italian ethic guidelines and regulations. Sections (3 μm thick) were deparaffinized and were incubated overnight at 4°C with primary antibodies (Table 2) and 3,3′-diaminobenzidine (DAB; Thermo Fisher Scientific) used as a chromogen. Sections were counterstained with hematoxylin and the percentage of stained area was evaluated with ImageJ 1.53k software. Representative photographs are shown (original magnification, 40X). Scale bar, 100 μm.

*Invasion assay*

A375 or SK-Mel-5 melanoma cells ($1 \times 10^4$ cells/well) were seeded in DMEM supplemented with 10% Bovine serum albumin (BSA), in the presence or absence of neutralizing antibodies onto the top chamber of 48-well transwell plates equipped with 8 μm polycarbonate nucleopore filters (Neuro Probe, Gaithersburg, MD, USA) pre-coated with Matrigel (Sigma-Aldrich). The bottom chamber was supplemented with CM obtained as described above. After 24 hour-incubation cells that had not migrated were removed with a cotton swab from the upper surface of filters and cells that had migrated to the lower surface of the membrane were subjected to Diff-Quick staining (Medion

Diagnostics AG, Dudingen, Switzerland) and observed with a light microscope. The number of cells per well was evaluated by counting cells in 5 randomly chosen microscope fields (20X magnification).

*Statistical analysis*

Data represent mean or ± SD values calculated on at least three independent experiments. P values were calculated using Student t-test or one-way ANOVA (multiple comparison). $P < 0.05$ was considered statistically significant.

## Results

*ERK5 inhibition determines an increase of LTBP1*

CXCL1, CXCL8 and CCL20 have been recently identified among the SASP-related soluble mediators that are responsible for the reduced proliferation in BRAFV600E melanoma cells undergoing cellular senescence following ERK5 KD [9]. In view of the exploitation of ERK5 targeting for the treatment of melanoma, further characterization of the secretome of BRAFV600E melanoma cells upon ERK5 inhibition was performed, taking advantage of a previously performed transcriptomic analysis in A375 and SK-Mel-5 ERK5-KD cells [9]. Using this approach, it emerged that ERK5 KD upregulated LTBP1 mRNA levels, when compared to control non-targeting shRNA-transduced cells (Supplementary Figure S1). QPCR confirmed the increased expression of LTBP1 mRNA upon ERK5 KD in both A375 and SK-Mel-5 BRAFV600E cells (Figure 1A). Interestingly, publicly available Skin Cutaneous Melanoma (SKCM) data set from TCGA on TNMplot [28] provided evidence that LTBP1 mRNA levels are lower in primary and metastatic melanomas than in normal tissues from non-cancer patients (Figure 1B). However, the same data set did not provide evidence of changes in ERK5 mRNA along melanoma progression (not shown), in keeping with a previous report showing consistent activation of the MEK5/ERK5 pathway without appreciable ERK5 overexpression in melanoma patients [8]. More importantly, using the same dataset on

cBioPortal for Cancer Genomics it emerged that higher expression levels of LTBP1 mRNA are associated with a better overall survival (OS) (Figure 1C) of melanoma patients, pointing to a possible tumor suppressive role of LTBP1 in melanoma. ERK5 KD resulted in increased levels of LTBP1 protein (Figure 1D), and the same effects were recapitulated by pharmacological inhibition of ERK5 using XMD8-92 [21] and the more specific JWG-071 [22] small molecule inhibitors (Figure 1E). Effectiveness of the ERK5 inhibitors was confirmed by the reduced protein level of the downstream target KLF2 [29]. Of note, AX15836 that inhibits the catalytic function of ERK5 but paradoxically stimulates its transactivation function [30,31] did not elicit the same effects (not shown). Taken together, the above data indicate that ERK5 negatively regulates LTBP1, whose expression correlates with a better outcome in melanoma patients.

*ERK5 inhibition promotes an LTBP1-dependent increase of TGF-β1, whose expression is associated with a better prognosis in melanoma*

Because LTBP1 is involved in the stabilization and activation of TGF-β, which plays a relevant role in cancer onset and progression [15], the impact of ERK5 KD on TGF-β protein levels was analyzed. Increased protein levels of both mature (Figure 2A) and latent forms (Supplementary Figure S2A) of TGF-β1 were found, in conditioned media (CM) and whole cell lysates, respectively, of ERK5-KD A375 and SK-Mel-5 cells. Of note, mRNA levels of TGFB1 were not consistently affected (i.e. were not increased in both cell lines upon ERK5 KD), pointing to post-transcriptional effects of LTBP-1 on TGF-β upon ERK5 KD (Supplementary Figure S2B), at least in our experimental models. Interestingly, in line with the fact that LTBP-1-dependent regulation of TGF-β could impact the activity of transcription factors (e.g. SMAD proteins, AP-1, NF-kB, and SP1 [32,33,34]) known to be regulated by TGF-β itself that are, in turn, able to regulate TGF-β expression, the SKCM data set from TCGA on cBioPortal provided evidence of a positive correlation (Spearman: 0.32, p = 2.82e-12) between LTBP1 mRNA and that of TGFB1 (Figure 2B). More importantly, the same dataset provided a positive association between higher levels of TGFB1 expression and a better

prognosis in melanoma patients (Figure 2C). To prove that LTBP1 participates in the regulation of TGF-β1 protein level in melanoma cells, LTBP1 was KD using two different siRNAs (Figure 2D). LTBP1 KD determined a marked decrease of TGF-β1 protein in both A375 and SK-Mel-5 cells (Figure 2E), and prevented the increase of TGF-β1 upon pharmacological inhibition of ERK5 (Supplementary Figure S2C). Importantly, both LTBP1 and TGF-β1 protein levels were increased upon ERK5 inhibition *in vivo*. Indeed, administration of the ERK5 inhibitor XMD8-92, which had been previously shown to reduce melanoma tumor growth similarly to ERK5 KD [8], induced a robust increase of both LTBP1 and TGF-β1 in A375 xenografts, with respect to vehicle-treated mice (Figure 2F).

*TGF-β1 exerts an antiproliferative effect in melanoma cells upon ERK5 KD*

It has been shown that ERK5 KD results in the increased production of CXCL1, CXCL8 and CCL20 in melanoma cells, and that these chemokines are responsible for a reduced viability of melanoma cells [9]. TGF-β is involved in cellular senescence and is able to exert potent growth inhibitory activities in various cell types and in different context, including cancer cells [13]. Along this line, here TGF-β1 emerges to be among the soluble factors responsible for a reduced viability of melanoma cells upon ERK5 KD. Indeed, TGF-β1-neutralizing antibodies prevented in a dose-dependent manner the anti-proliferative effect induced by CM harvested from ERK5-KD A375 (Figure 3A) or SK-Mel-5 (Figure 3B) cells. The above effects were not restricted to BRAFV600E-mutated melanoma cells. Indeed, in both N-RAS-mutated SK-Mel-2 and triple wild type SSM2c melanoma cells, TGF-β1-neutralizing antibodies reverted the reduction of cell proliferation elicited by the ERK5-KD-derived CM (Supplementary Figure S3A). Moreover, in keeping with the biological evidence, both pharmacological and genetic inhibition of ERK5 determined an increase of TGF-β1 in these cell lines (Supplementary Figure S3B). To confirm that TGF-β1 exerts an antiproliferative effect in melanoma cells, A375 and SK-Mel-5 cells were treated with human recombinant TGF-β1.

This cytokine reduced the number of viable cells in culture in a dose-dependent manner in both cell lines (Figure 3C). This effect was maximal with 100 ng/ml, a concentration in line with previous reports [35,36]. In order to deepen how TGF-β1 affects cell growth, cell-cycle analysis was performed, and showed that treatment with TGF-β1 significantly increased the fraction of cells in G0/G1 phase (Figure 3D). In the same experimental settings, TGF-β1 determined the increase of the cyclin dependent kinase inhibitor p21 (Supplementary Figure S3C). In further support of a possible involvement of p21 in the antiproliferative effects of TGF-β1 upon ERK5 inhibition, the treatment with TGF-β1-neutralizing antibodies was able to reduce the increase of p21 elicited by CM harvested from ERK5-KD (A375) cells in both A375 and SK-Mel-5 cell lines (Supplementary Figure S3D). The reduction of cell number observed in melanoma cells treated with TGF-β1 was partially due to increased cell death (Figure 3E). On the whole, the above data provides evidence that TGF-β1 is among the soluble mediators that increase upon ERK5 inhibition, and is then responsible for the reduced proliferation.

*TGF-β1 produced upon ERK5 inhibition reduces the invasive ability of melanoma cells*

The possible impact of the secretome of ERK5-KD cells on melanoma cell invasiveness was then tested. CM from ERK5-KD cells markedly reduced the invasive ability of A375 and SK-Mel-5 cells (Supplementary Figure S4A), in the presence of mimosine, a DNA replication inhibitor used at concentration able to completely prevent changes in the number of cells along the duration (i.e. 24 hours) of the invasion assays (Supplementary Figure S4B and C). To shed light on the possible role of TGF-β1 in the regulation of this biological process upon ERK5 KD, the effect of TGF-β1 neutralizing antibodies on cell invasion ability was evaluated. TGF-β1 neutralizing antibodies were able to restore A375 (Figure 4A) and SK-Mel-5 (Figure 4B) invasion ability reduced by CM harvested from ERK5-KD A375 or SK-Mel-5 cells, while control IgG did not. To confirm that TGF-β1 is able to reduce melanoma cell invasiveness, A375 and SK-Mel-5 cells were treated with increasing doses

of this cytokine. TGF-β1 dose-dependently decreased the invasive ability of both A375 and SK-Mel-5 cells (Figure 4C and D). Altogether, the above data indicate that TGF-β1 reduces the invasive propensity of melanoma cells, at least *in vitro*.

*Increased TGF-β1 and LTBP1 expression positively affects the impact of immunotherapy in melanoma patients*

As reported above, higher levels of both TGF-β1 and LTBP1 correlate with a better OS (Figure 1C and 2C). Moreover, OS and disease-free survival (DFS) of patients treated with anti-PD1 therapy (i.e. Nivolumab or Pembrolizumab) are significantly higher in patients with high TGF-β1 expression than in those with lower expression (Figure 5A). This positive association was also detected regarding high levels of LTBP1 expression and better OS and DFS in melanoma patients treated with anti-PD1 therapy (Figure 5B), pointing to additional desirable effects of ERK5 inhibition in melanoma.

## Discussion

TGF-β controls a wide spectrum of cellular functions, and deregulated TGF-β signaling has been linked to several human diseases, including cancer [37]. In particular, TGF-β may play a double-edged sword role in tumor progression [38,39], acting as a tumor suppressor during the early stage of the tumor, since inhibition of TGF-β signaling results in the disruption of normal homeostatic process and subsequent carcinogenesis, while behaving as a tumor promoter at later stages [40]. Understanding how TGF-β1 can coordinate its effects in melanoma is a key issue in the biology of this cancer.

ERK5 has been recently reported to be involved in melanoma growth [8], and ERK5 inhibition induces marked cellular senescence and production of several soluble mediators involved in the SASP in both BRAF-mutated and -wild-type melanoma cells and xenografts [9]. In this study, ERK5 inhibition evokes an increased expression of LTBP1, which is known to modulate the availability of

316 TGF-β1 [15]. Along this line, besides increased LTBP1 expression, increased TGF-β1 protein levels
317 were found in ERK5-KD melanoma cells, and in A375 xenografts from XMD8-92-treated mice.
318 LTBP1 resulted to be responsible for the regulation of TGF-β1 protein levels, likely through a post
319 transcriptional regulation, and also to prevent the increase on the latter upon ERK5 inhibition. This
320 work also identifies an anti-proliferative and anti-invasiveness ability of TGF-β1 in melanoma cells,
321 providing evidence that the increase of LTBP1/TGF-β1 complex could be an additional desirable
322 effect obtained by ERK5 inhibition.

323 TGF-β is a potent inhibitor of cell proliferation, which is thought to result from its ability to
324 induce G1 cell cycle arrest [41]. In line with this fact, the data provided in this work indicate that
325 TGF-β1 is among the soluble factors responsible for the reduction of melanoma cell proliferation
326 induced by the secretome of ERK5-KD melanoma cells. In fact, this event is partially restored by
327 TGF-β1-neutralizing antibodies. Moreover, in BRAFV600E-expressing cells, TGF-β1 slows down
328 cell cycle progression with the accumulation of cells in G0/G1 phase, and is able to increase cell
329 death. Despite these effects are elicited at relatively high TGF-β1 concentrations, the latter are in line
330 with previous reports [35,36], and are consistent with the amount contained in the CM of ERK5-KD
331 melanoma cells. The observed antiproliferative effects are consistent with the results obtained in other
332 studies, which demonstrated that cell cycle arrest was induced upon treatment with TGF-β1 via
333 SMAD2/3 in proliferating melanoma cells *in vitro* and *in vivo* [42,43,44]. Moreover, in another paper,
334 the activation of TGF-β1 led to the upregulation of PAI-1 expression that resulted in tumor growth
335 inhibition in murine melanoma [45]. The above results, including those described in this manuscript,
336 are at variance with a previous report showing that inhibition of canonical TGF-β signaling inhibited
337 tumor growth in melanoma [46]. Despite here it clearly emerges an oncoppressive role for TGF-β1
338 in melanoma cells upon ERK5 inhibition, the molecular mechanism underlying this connection
339 remains to be established. However, TGF-β1 was found to increase the expression of the cyclin

dependent kinase inhibitor p21, a previously established ERK5-regulated protein [8,9], that is a downstream mediator of the antiproliferative effects of TGF-β, including in melanoma cells [38,47].

Another interesting finding of this study is the demonstration that ERK5-KD melanoma cells produce TGF-β1, which exerts an anti-invasive capacity. These results, together with the identified antiproliferative effect, are in keeping with the evidence reported here that melanoma patients with higher expression of TGF-β1 have a better prognosis. On the other hand, they are at variance with the established notion that, at least in the advanced stages, TGF-β acts as a tumor promoter by stimulating invasiveness along the epithelial to mesenchymal transition [48]. Of note, A375 and SK-Mel-5 cell lines used as models for this study were derived from metastatic melanoma [18,19]. Moreover, elevated expression levels of TGF-β1 have been associated with melanoma progression *in vivo*, and TGF-β1-elicited signals have been reported to stimulate melanoma cell dissemination from primary tumors [49,50].

From the clinical point of view, the possibility to elicit an increase in LTBP1 and TGF-β1 expression following ERK5 inhibition seems to have positive therapeutic implications in melanoma patients. Indeed, LTBP1 expression is lower in primary and metastatic melanoma compared to healthy tissues, and melanoma patients with higher expression of LTBP1 or TGF-β1 have a better prognosis (OS) with respect to those with lower ones. On the other hand, *in silico* data analysis revealed that among melanoma patients that have received anti-PD1 antibodies, those with higher expression of LTBP1 or TGF-β1 showed improved OS or DFS compared to those with low expression. This fact is of relevance, taking into consideration that TGF-β affects multiple components of the immune system, exerting most of times systemic immune suppression [51]. Furthermore, the first-line therapeutic approach for advanced melanoma consists in immunotherapy with anti-PD1 antibodies (Nivolumab/Pembrolizumab) or targeted therapy with BRAF and MEK inhibitors, and their combination is under study [52]. Based on all above, targeting ERK5 is also expected to boost the efficacy of immunotherapy in melanoma patients, adding value to the possible

targeting of ERK5 in this cancer, taking into consideration that ERK5 inhibition has been reported to reduce melanoma growth and to improve BRAF targeting *in vivo* [8], and that ERK5 activation is among the resistance mechanism to RAF-MEK1/2-ERK1/2 directed therapies [9].

**Authors' Contributions**

AT: Data curation, formal analysis, investigation, writing–original draft, writing–review and editing. AM, ZL: Data curation, formal analysis, methodology, writing–review. IT: Data curation, formal analysis and editing. TG: formal analysis, methodology. AE-O, AP, BS Resources and editing. ER: Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, investigation, writing–original draft, project administration, writing–review and editing.

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518 **Figure Legends**

519 **Figure 1. Effects of ERK5 inhibition on the expression of LTBP1 in melanoma cells. A**)
520 A375 and SK-Mel-5 cells transduced with lentiviral vectors harboring control non-targeting shRNA
521 (shNT) or ERK5-specific shRNAs (shERK5-1 and shERK5-2) were lysed after 72 hours, and LTBP1
522 mRNA levels determined by qPCR. Data shown are means (± SD) from three independent
523 experiments. **, $p < 0.01$ *vs* shNT. **B)** Violin plots show LTBP1 gene expression profile in normal
524 skin (Normal), primary (Tumor) and metastatic (Metastatic) melanoma obtained by SKCM data set
525 (TCGA) on TNMplot. ****, $p<0.0001$. **C)** Kaplan-Meier analysis of the relationship between LTBP1
526 expression and overall survival (OS) in melanoma patients using the SKCM data set (TCGA) on
527 cBioPortal. Patients were stratified according to low or high LTBP1 expression. Median LTBP1
528 expression value was used as cut-off. In order to reduce noise, 5% of samples above and below the
529 cut-off value were excluded from the analysis (n=423, with n=223 and n=200 in the low/high group,
530 respectively). HR = hazard ratio; HR < 1 indicates reduced hazard of death. **D**) A375 and SK-Mel-5
531 cells transduced with control shNT or shERK5 (shERK5-1 and shERK5-2) were lysed after 72 hours.
532 Western Blot was performed with the indicated antibodies. Images are representative of three
533 independent experiments showing similar results. Migration of molecular weight markers is indicated
534 on the left (kDa). The graphs show average relative integrated density (RID) ± SD of ERK5 protein
535 levels normalized for tubulin content from three independent experiments. *, $p <0.05$, **, $p < 0.01$ *vs*
536 shNT. **E**) A375 and SK-Mel-5 cells treated with XMD8-92 (5 μM) or JWG-071 (5 μM) for 72 hours
537 were lysed. Western Blot was performed with the indicated antibodies. Images are representative of
538 three independent experiments showing similar results. Migration of molecular weight markers is
539 indicated on the left (kDa). The graphs show average relative integrated density (RID) ± SD of LTBP1
540 protein levels normalized for tubulin content from three independent experiments. *, $p <0.05$, **, p
541 $< 0.01$ *vs* Vehicle.
542

**Figure 2. Effects of ERK5 inhibition on TGF-β1 expression in melanoma cells and in xenografts. A**) TGF-β1 immunoprecipitation was performed in 72-hour-conditioned media (CM) from A375 or SK-Mel-5 cells transduced with shNT or shERK5 (shERK5-1 or shERK5-2) lentiviral vectors. Human recombinant TGF-β1 (100 ng/ml) was used as positive control. Western Blot was performed with the indicated antibodies. Images are representative of three independent experiments showing similar results. Migration of molecular weight markers is indicated on the left (kDa). The graphs show average relative integrated density (RID) ± SD of TGF-β1 protein levels normalized for IgG content from three independent experiments. *, $p < 0.05$, **, $p < 0.01$ *vs* shNT. **B)** Expression levels of TGFB1 and LTBP1 mRNA from SKCM dataset (TCGA) on cBioPortal. **C)** Kaplan-Meier analysis of the relationship between TGFB1 expression and overall survival (OS) in melanoma patients using SKCM dataset (TCGA) on cBioPortal. Patients were stratified according to low or high *TGFB1* expression, using median TGFB1 expression value as cut-off. In order to reduce noise, 10% of samples above and below the cut-off value were excluded from the analysis (n=376, with n=195 and n=181 in the low/high group, respectively). HR= hazard ratio; HR < 1 indicates reduced hazard of death. **D**) QPCR of LTBP1 mRNA from LTBP1-KD A375 and SK-Mel-5 cells following treatment with LTBP1-targeting (siLTBP1-1 or siLTBP1-2) siRNAs or control non-targeting siRNAs (siNT) for 72 hours. Data shown are mean (±SD) of three independent experiments. *, $p < 0.05$, **, $p < 0.01$ *vs* siNT. **E)** Western Blot showing TGF-β1 protein levels in LTBP1 KD-cells 72 hours after transfection with LTBP1-targeting siRNA (siLTBP1-1 or siLTBP1-2) or control non-targeting siRNA (siNT). Migration of molecular weight markers is indicated on the left (kDa). The graphs show average relative integrated density (RID) ± SD of TGF-β1 protein levels normalized for tubulin content from three independent experiments. *, $p < 0.05$ *vs* siNT. **F)** IHC detection of LTBP1 (left) or TGF-β1 (right) in XMD8-92 (25 mg/kg)- or vehicle (2-hidroxypropyl-β-cyclodextrin 30%)-treated mice [8]. Hematoxylin counterstaining was performed. Bar plots of percentage (%) of LTBP1 or TGF-β1-positive cells are shown. The percentage of positive cells was calculated from six different ×40 magnified fields from three randomly chosen vehicle and XMD8-92-treated tumors.

569 Representative photographs are shown (original magnification, ×40). Scale bar, 100 μm. *, p <0.05
570 *vs* Vehicle.
571
572 **Figure 3. Involvement of TGF-β1 in the anti-proliferative outcome of ERK5 inhibition**
573 **in melanoma cells. A-B**) MTT performed in A375 and SK-Mel-5 cells treated for 72 hours with 72-
574 hour conditioned media (CM), obtained from A375 or SK-Mel-5 cells transduced with shNT or
575 shERK5 (shERK5-1 or shERK5-2) lentiviral vectors, alone or in combination with the indicated
576 concentrations of TGF-β1 neutralizing antibodies (TGF-β1 Neu-Ab). Data shown are means (± SD)
577 from three independent experiments. *, p <0.05, **, p < 0.01 *vs* shNT CM; §, p<0.05, §§, p<0.01 *vs*
578 shERK5-1 CM; #, p<0.05 *vs* shERK5-2 CM. **C)** Cells were treated with the indicated concentrations
579 of TGF-β1 for 72 hours, and the number of viable cells was counted. Histograms represent means
580 (±SD) from three independent experiments. **p < 0.01 *vs* untreated. **D)** Cells were treated or not with
581 100 ng/ml human recombinant TGF-β1 for 72 hours, and cell-cycle phase distribution was then
582 determined. Data shown are means ± SD from three independent experiments. *p < 0.05 *vs* untreated.
583 **E)** Dead cells (Annexin-V-positive and Annexin-V-negative/PI-positive cells) were evaluated after
584 treating A375 or SK-Mel-5 melanoma cells with or without 100 ng/ml of human recombinant TGF-
585 β1. Histograms represent mean percentages ± SD from three independent experiments. *p < 0.05 *vs*
586 untreated.
587
588 **Figure 4. Involvement of TGF-β1 in the anti-invasive effect of ERK5 inhibition in**
589 **melanoma cells. A-B**) Invasion assays were performed for 24 hours in A375 (**A**) and SK-Mel-5 (**B**)
590 cells in the presence of 72-hour CM, obtained from A375 or SK-Mel-5 cells transduced with shNT
591 or shERK5 (shERK5-1 or shERK5-2) lentiviral vectors, alone or with TGF-β1 neutralizing antibodies
592 (TGF-β1 Neu-Ab, 10 μg/ml) or control IgG. Histograms represent means (± SD) from three
593 independent experiments. **p < 0.01 *vs* shNT CM/0, ##p<0.01 *vs* shERK5-1 CM/0, §§p<0.01 *vs*
594 shERK5-2 CM/0; ns: not significant. Representative pictures of wells treated as above are included.

595 Scale bar, 150 µm. **C-D)** A375 and SK-Mel-5 cells were exposed for 24 hours at increasing
596 concentrations of human recombinant TGF-β1. Histograms represent means (± SD) from three
597 independent experiments. **p < 0.01 *vs* NT.
598
599 **Figure 5. Impact of LTBP1 and TGF-β1 expression on the overall survival and disease-**
600 **free survival in anti-PD1-treated melanoma patients. A)** 60 months follow-up Kaplan-Meier
601 analysis of the relationship between TGF-β1 expression and overall survival (OS) in anti-PD1-treated
602 melanoma patients (n=325) from Kaplan-Meier plotter database. Patients were stratified according to
603 low or high TGF-β1 expression. The number of patients at risk in the low and high expression groups
604 are indicated. **B)** 60 months follow up Kaplan-Meier analysis of the relationship between TGF-β1
605 expression and DFS in melanoma anti-PD1 treated patients (n=234) calculated as in A. **C)** 60 months
606 follow up Kaplan-Meier analysis of the relationship between LTBP1 expression and OS in melanoma
607 anti-PD1 treated patients (n=325) calculated as in A. **D)** 60 months follow up Kaplan-Meier analysis
608 of the relationship between LTBP1 expression and DFS in melanoma anti-PD1 treated patients
609 (n=200) calculated as in A.
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**Table 1**. List of the antibodies used and their application.
WB: Western Blot; IHC: Immunohistochemistry; N: Neutralization.

| | | | | |
|---|---|---|---|---|
| ERK5 | WB | Rabbit polyclonal | #3372 | Cell Signaling Technology, USA |
| LTBP1 | WB, IHC | Mouse monoclonal | sc-271140 | Santa Cruz Biotechnology, USA |
| α-Tubulin | WB | Mouse monoclonal | sc-32293 | Santa Cruz Biotechnology, USA |
| TGF-β | WB | Rabbit polyclonal | #3711 | Cell Signaling Technology, USA |
| TGF-β1 | N, IHC | Mouse monoclonal | 69012-1-Ig | Proteintech Group, Inc, USA |
| KLF-2 | WB | Rabbit monoclonal | #15306 | Cell Signaling Technology, USA |
| IgG1 | N | Mouse monoclonal | MAB002 | R&D Systems, Inc. USA |

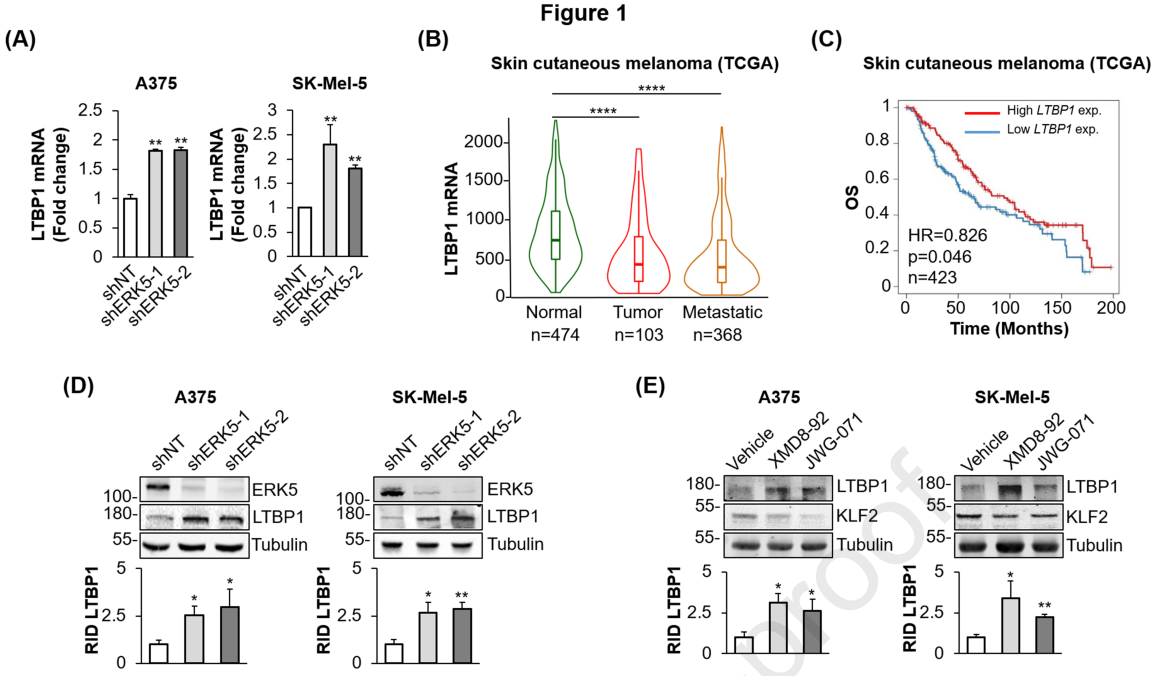
644 **Table 2.** List and sequences of the shRNA. *****Sequence reference from
645 https://www.ncbi.nlm.nih.gov/gene/5598

| Gene | Sequence reference* | shRNA | Clone ID | Sequence |
|---|---|---|---|---|
| none | none | shNT | SHC202 | 5'-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT-3' |
| *MAPK7* | NM_139032.X | shERK5-1 | TRCN0000010262 | 5'-CCGGGCTGCCCTGCTCAAGTCTTTGCTCGAGCAAAGACTTGAGCAGGGCAGCTTTTT-3' |
| *MAPK7* | NM_139032.X | shERK5-2 | TRCN0000010275 | 5'-CCGGGCCAAGTACCATGATCCTGATCTCGAGATCAGGATCATGGTACTTGGCTTTTT-3' |

646

647

Figure 1



## Figure 2

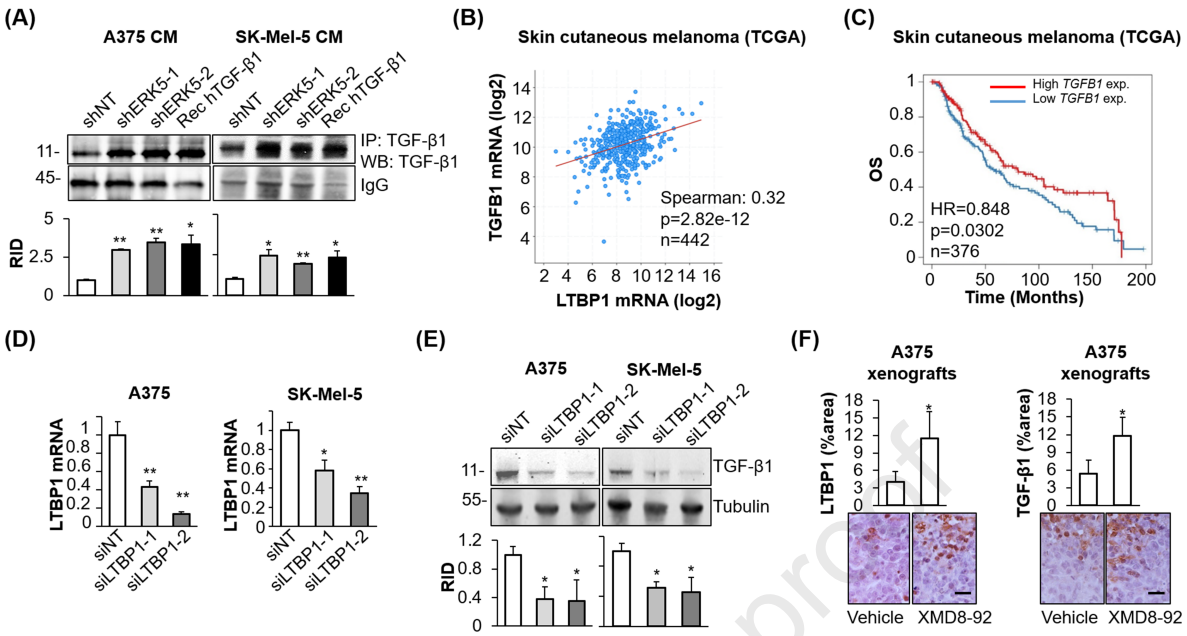


**Figure 3**

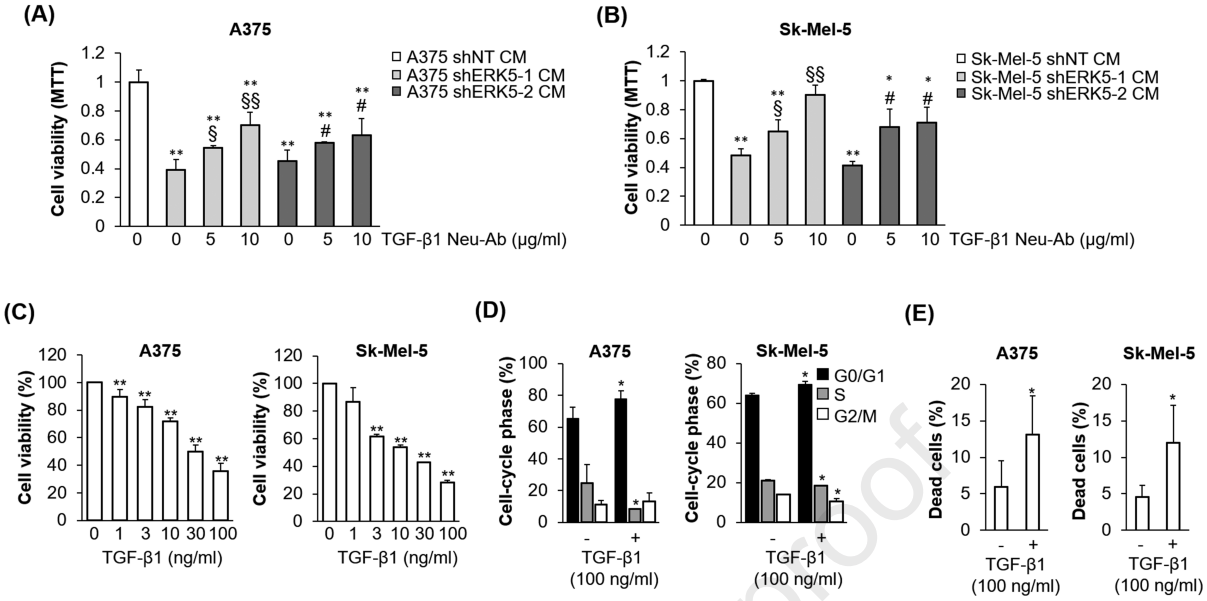
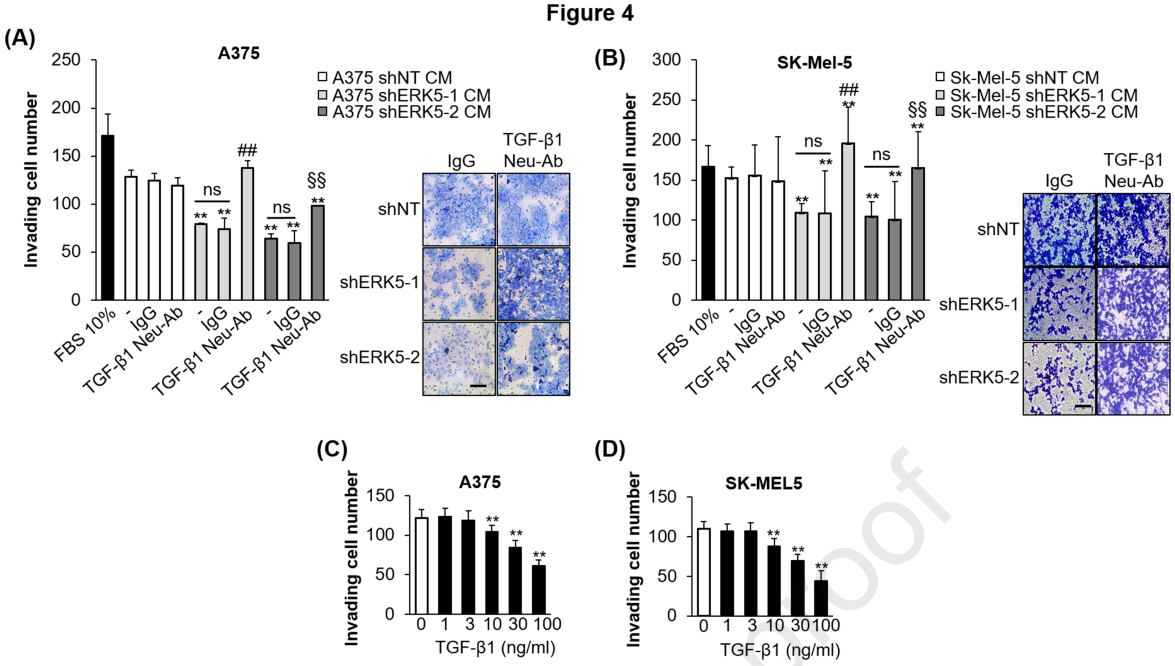


**Figure 4**



## Figure 5